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Long-Term Culture of Freshwater Mussel Gill Strips: Use of Serotonin to Affect Aseptic Conditions

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Abstract. Serotonin relaxes the musculature and increases epithelial ciliary activity in freshwater mussel gills. This results in greater than normal water flow through the labyrinth of water canals and channels of the gill. These water spaces harbor significant microbial populations that make aseptic culture of freshwater mussel gills difficult. High concentrations of antibiotics can maintain short-term cultures, but are toxic to the tissue and reduce the lifespan of the culture. Moderate levels of antibiotics used in combination with 0.1 m*M* serotonin during a single, short pretreatment produces aseptic cultures. These cultures can now be established routinely and are viable for over a month as assayed by gill structural integrity, trypan blue exclusion, leucine incorporation into TCA precipitable protein, and normal physiological responsiveness to serotonin re-exposure.

Introduction

Attempts at establishing molluscan organ cultures have been made since the mid-1920's. Zweibaum (1925) tried to culture freshwater mussel gills in various diluted vertebrate Ringer's solutions enriched with peptone. Mantle tissue explants, from the land snail *Helix aspersa* (Gatenby, 1931; Gatenby *et al.*, 1934) and the marine bivalve *Pinctata* (Bevelander and Martin, 1949), were cultured. Cultured explants of the heart, lungs, and foot of various *Helix* species have also been reported (Konicek, 1933). Unfortunately, these organ cultures remained functional for only a few days.

More recently, certain cells and tissues, particularly of pulmonate gastropods and some marine organisms, have been successfully cultured. The cell lines cultured were derived from several tissues: ganglia, gills, heart, digestive tract, foot, mantle, oviduct, connective tissue, and gonad (Burch and Cuadros, 1965; Cheng and Arndt, 1973; Hansen, 1975; Kaczmarek *et al.*, 1979; Sengal, 1961a,b; Stephens and Hetrick, 1979; Vago and Chastang, 1958). Unfortunately, attempts at culturing freshwater mussel tissue have failed due to fungal and bacterial contamination (Sengel, 1964), and the lack of well-defined maintenance media.

The gills of freshwater mussels are complex organs that function in ion transport, respiration, food capture and sorting, storage and maintenance of embryos during reproduction, calcium storage, and water movement. Several different cell types are associated with the gill, but culturing gill explants or sub-culturing specific cell lines allows the study of tissue- and cell-specific responses of the gill in isolation from the rest of the organism. Previously we have studied gills excised from the animal immediately before use (Dietz and Findley, 1980; Dietz *et al.*, 1982; Dietz and Hagar, 1990; Silverman *et al.*, 1991). Having viable gill explants functioning in culture expands the range and duration of experiments that can be accomplished.

Here we report the successful long-term culture of freshwater mussel gills under aseptic conditions. A suitable medium, methods for reducing antibiotic exposure, and culture viability (as judged by normal appearance and physiological responses in gill tissue) are documented.

Materials and Methods

Animals and maintenance

Freshwater mussels *Anodonta grandis* and *Ligumia subrostrata* were collected from shallow ponds near Baton Rouge, Louisiana. The animals were maintained in aerated artificial pond water (Table 1) at 22–25°C, and were

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Abbreviations: Penicillin (Pen); streptomycin (Strep); amphotericin B (Am-B); Artificial mussel hemolymph (AMH).

allowed to acclimate to laboratory conditions for a week before use.

Initial short-term culture methods

Gills were washed (3 × 20 min) in sterile pondwater or 30 m*M* Tris-HCl, pH 7.8, containing up to 1500 U ml^{-1} penicillin G (Pen) and 1.5 mg ml^{-1} streptomycin (Strep). The preliminary media we tested contained multiple components chosen for their presence in diluted vertebrate culture media, or in mussel hemolymph (Table I). As the major source of the defined nutrients, we selected either a medium based on vertebrate Ham's F-12 (Gibco) (dilution I), or artificial mussel hemolymph (AMH). All cultures contained 1 mg l^{-1} phenol red, which allowed us to monitor pH. Cultures in each medium appeared normal and were viable for 1–4 days, but microbial and fungal contamination remained a problem.

Antibiotic treatments

To reduce fungal contamination, we exposed gill strips to amphotericin B (Am-B) in concentrations ranging from 25 to 500 $\mu g\ ml^{-1}$ and for exposure times of from 1 to 24 h. We also pretreated intact animals in solutions of antibiotics at concentrations patterned after those used by Stephens and Hetrick (1979) to decontaminate oyster tissues. For such whole animal treatment, the following antibiotics were added to mussel Ringer's or pondwater (Table I): 1000 U ml^{-1} penicillin G and 1 mg ml^{-1} streptomycin, 100 $\mu g\ ml^{-1}$ neomycin, 50 $\mu g\ ml^{-1}$ chlortetracycline, 125 $\mu g\ ml^{-1}$ gentamicin, 500 $\mu g\ ml^{-1}$ kanamycin sulfate, 500 $\mu g\ ml^{-1}$ polymyxin B sulfate, 100 $\mu g\ ml^{-1}$ erythromycin, 25 $\mu g\ ml^{-1}$ amphotericin B, and 250 U ml^{-1} nystatin. The mussels were immersed in this solution for 3–4 days. Gills were removed from both pretreated and non-pretreated clams, cut into 3–5 mm strips, and cultured as described above.

Serotonin application

Serotonin enhances the ciliary activity and relaxes the muscles of the gills in freshwater mussels (Gardiner *et al.*, 1991), so we used it as an aid in decontamination. Gills were isolated and placed in sterile tubes containing 10^{-4} *M* serotonin in freshwater mussel Ringer's, pH 7.8. The solution was changed every 5 min for 20 min. The gills were removed and placed in sterile tubes containing freshwater mussel Ringer's with 500 U ml^{-1} penicillin G, 0.5 mg ml^{-1} streptomycin, 500 $\mu g\ ml^{-1}$ colistin, 10 $\mu g\ ml^{-1}$ chlortetracycline, 250 U ml^{-1} nystatin, and 10^{-4} *M* serotonin, pH 7.8. This solution was replaced every 15 min for a total incubation period of 45 min. Gills were cut into 3–5 mm strips that were distributed in 24-well Corning culture plates containing an enriched Ham's F-12 medium (Table I, Ham's F-12 dilution II) or artificial mussel hemolymph (AMH, Table I). Both media were fortified with 500 U ml^{-1} penicillin G, 0.5 mg ml^{-1} streptomycin, and 5 $\mu g\ ml^{-1}$ chlortetracycline, pH 7.8. To some of these cultures, 250 U ml^{-1} nystatin also was added. The pH was adjusted to 7.8, and the osmolality adjusted to 60 mosm (Precision System Micro Osmometer), as needed. The explants were maintained at 20–23°C, and the explant was placed in a new well with fresh medium every two days.

Assessment of tissue viability

The initial assessment of viability was by observation, with an inverted microscope, of explant integrity, periodic muscular contractions, and ciliary activity. Trypan blue exclusion was used to determine cellular viability (Freshney, 1987). Cells detached from the explant were placed in a 0.2% trypan blue in mussel Ringer's for 5 min. Cells were viewed under a light microscope, and dying ones were identified by their uptake of trypan blue.

Serotonin (10^{-5} *M*) increases the gill ciliary activity and relaxes gill musculature. We used these responses to assay for the proper physiological response of the explants. The explants were placed into fresh medium containing serotonin and observed for 5 min for muscular reflex activity and changes in the pattern of ciliary motion (Gardiner *et al.*, 1991).

Several cultures were assayed for their ability to incorporate ^{3}H-leucine into a trichloroacetic acid (TCA) precipitable protein fraction. Approximately 1 $\mu Ci\ ml^{-1}$ ^{3}H-leucine (specific activity 1 mCi μM^{-1} leucine) was added to AMH culture medium in which the leucine concentration had been reduced to 1 μM. After a 1-h exposure, the gills were denatured with 10% TCA, rinsed in pondwater, blotted, and their wet tissue weights recorded. The gills were homogenized in 1 ml 10% TCA and centrifuged at 5000 × g for 5 min. The pellet was twice resuspended and centrifuged in 10% TCA. The pellet was dissolved in 1 *M* NaOH, and the radioactivity was determined in a liquid scintillation counter. Protein concentration was determined by the method of Bradford (1976).

Analysis of microbial contaminants

Fungi growing in the cultures were identified only by the appearance of structural hyphae; no attempt was made to identify the species. The bacterial contaminants surviving antibiotic treatment were cultured in antibiotic-free Ham's F-12 dilution II (Table I) and sent to Louisiana State Veterinary Medical Diagnostic Laboratory for identification.

Results

Our initial attempts to establish sterile gill explants in culture, using a variety of antibiotics, were unsuccessful.

Table 1

Composition of media used for culturing freshwater mussel gills

	Pond water	FW mussel Ringer's	Ham's F-12 dilution I	Ham's F-12 dilution II	Art. mussel hemolymph	FW mussel hemolymph
Inorganic salts (m*M*)						
$CaCl_2$	0.40	5.0	0.04	0.03	4.79	a, b, c
$CuSO_4 \cdot 5H_2O$	0	0	1.4×10^{-7}	1.0×10^{-7}	0	—
$FeSO_4 \cdot 7H_2O$	0	0	4.2×10^{-4}	2.99×10^{-4}	0.028	d
KCl	0.05	0.5	0.438	0.313	0	a, b, c
K_2HPO_4	0	0	0	0	0.197	a, b, c
$MgCl_2 \cdot 6H_2O$	0	0	0.09	0.061	0.187	b
$MnCl_2 \cdot 4H_2O$	0	0	0	0	0.12	d
NaCl	0.50	5.0	18.2	13.0	16.18	a, b, c
$NaHCO_3$	0.20	5.0	0	0	4.59	a, b, c
Na_2HPO_4	0	0	0.190	0.142	0	a, b, c
Na_2SO_4	0	5.0	0	0	0	b
$ZnSO_4 \cdot 7H_2O$	0	0	4.2×10^{-4}	3.0×10^{-4}	0.0048	d
Amino acids (μM)						
L-Alanine	0	0	14.0	10.0	55.0	55 ± 7 (4) e
L-Arginine	0	0	140.0	100.0	14.0	14 ± 4 (4) e
L-Asparagine	0	0	14.0	10.0	0	—
L-Aspartate	0	0	14.0	10.0	27.0	27 ± 10 (4) e
L-Cysteine	0	0	28.0	20.0	0	—
L-Glutamate	0	0	14.0	10.0	54.0	54 ± 16 (4) e
L-Glutamine	0	0	140.0	100.0	0	—
Glycine	0	0	14.0	10.0	12.0	12 ± 4 (4) e
L-Histidine	0	0	14.0	10.0	26.0	26 ± 4 (4) e
L-Isoleucine	0	0	4.2	3.0	8.0	8 ± 1 (4) e
L-Leucine	0	0	14.0	10.0	13.0	13 ± 1 (4) e
L-Lysine	0	0	28.0	20.0	15.0	15 ± 4 (4) e
L-Methionine	0	0	4.2	3.0	0	—
L-Phenylalanine	0	0	4.2	3.0	8.0	8 ± 1 (3) e
L-Proline	0	0	42.0	30.0	14.0	14 ± 1 (4) e
L-Serine	0	0	14.0	10.0	83.0	83 ± 7 (4) e
L-Threonine	0	0	14.0	10.0	134.0	59; 134 e
L-Tryptophan	0	0	1.4	1.0	0	—
L-Tyrosine	0	0	4.2	3.0	3.0	3.0 e
L-Valine	0	0	14.0	10.0	14.0	14 ± 2 (4) e
Vitamins (m*M*)						
Biotin	0	0	4.2×10^{-6}	3.0×10^{-6}	4.09×10^{-3}	—
Choline chloride	0	0	0.02	0.01	7.16×10^{-3}	—
Folic acid	0	0	4.2×10^{-4}	3.0×10^{-4}	2.26×10^{-3}	—
myo-inositol	0	0	0.014	0.01	0.011	—
Niacinamide	0	0	4.2×10^{-5}	3.0×10^{-5}	8.19×10^{-3}	—
Pantothenate (Ca)	0	0	1.4×10^{-4}	1.0×10^{-4}	2.09×10^{-3}	—
Pyridoxine	0	0	4.2×10^{-5}	3.0×10^{-5}	4.86×10^{-3}	—
Riboflavin	0	0	1.4×10^{-5}	1.0×10^{-5}	2.70×10^{-4}	—
Thiamine	0	0	1.4×10^{-4}	1.0×10^{-4}	2.96×10^{-3}	—
Vitamin B-12	0	0	1.4×10^{-4}	1.0×10^{-4}	0	—
Other (m*M*)						
Glucose	0	0	1.4	5.55	5.55	—
Fetal serum (v/v)	0	0	8.7%	5%	5%	—
Hemolymph (v/v)	0	0	8.7%	0	0	100%
Hypoxanthine	0	0	0.0042	0.003	0	—
Insulin (U ml^{-1})	0	0	0.25	0	0	—
Linoleic acid	0	0	4.2×10^{-5}	3.0×10^{-5}	0	—
Lipoic acid	0	0	1.4×10^{-4}	0	0	—
pH	7.0	7.8	7.8	7.8	7.8	7.8
Phenol red (mg l^{-1})	0	1.0	1.0	1.0	1.0	0
Putrescine	0	0	1.4×10^{-4}	1.0×10^{-4}	0	—
Pyruvic acid	0	0	0.15	0.1	0	—
TES buffer	0	0	2.0	0	0	—
Thioctic acid	0	0	0	1.0×10^{-4}	0	—
Thymidine	0	0	1.4×10^{-4}	3.0×10^{-4}	0	—

Refer to (a) Dietz (1979), (b) Potts (1954), (c) Silverman *et al.*, (1983), (d) Silverman *et al.*, (1987) for ionic composition and (e) Hanson and Dietz (1976) for amino acids in freshwater mussel hemolymph. Art. = artificial, FW = freshwater.

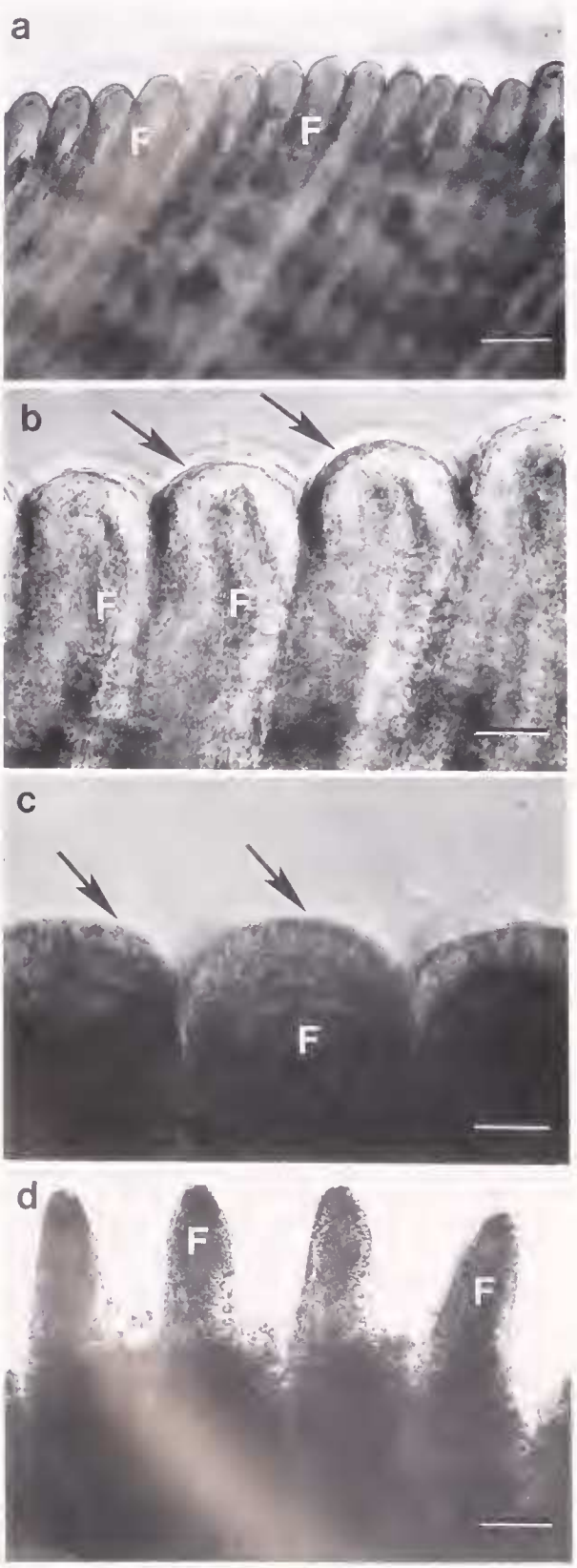

Figure 1. (a) Light micrograph of *Ligumia subrostrata* gill explant, in culture less than 24 h, showing normal filament (F) organization. (b) Higher magnification micrograph of (a) showing filaments (F) and active cilia (arrows) indicated by the refractile halo pattern. (c) Light micrograph of *Anodonta grandis* gill explant cultured for 30 days, showing filaments (F) and active cilia (arrows) as indicated by the halo pattern. (d) Light micrograph of *Anodonta grandis* gill explant maintained in culture for 40 days. Gill integrity has been compromised and cells have detached from explant. This detachment is mostly observable at the filaments (F). Bars (a) = 137 μm; (b) = 35 μm; (c) = 37 μm; (d) = 77 μm.

All of the explants remained functional for several days, but were contaminated by predominantly gram-negative bacteria of the genus *Leukothrix*, commonly associated with freshwater mussels and shrimp (Louisiana State Veterinary Medical Diagnostic Laboratory). In addition, the gill cultures treated with Am-B at concentrations up to 500 μg ml^{-1} for 12 h had fungi within a few days. High doses of Am-B caused copious mucus secretion and inhibition of ciliary activity. With the growth of fungi and bacteria, the culture medium rapidly acidified in spite of frequent (1–2 days) medium changes.

Treatment with high concentrations of antibiotics was ineffective; 75% of the cultures still contained fungal contamination, and all contained 2–3 strains of bacteria. Explants not contaminated with fungus remained viable for 10–14 days, but all had bacterial contamination. Pretreatment of the intact animal with antibiotics did not reduce microbial contamination. The microorganisms were unlikely to be resistant to the wide spectrum of antibiotics we used; we conclude rather that the gill was harboring the microorganisms in the extensive branchial canals and channels, isolating them from the antibiotics in the medium.

Serotonin pretreatment of gill explants

Explants treated with serotonin rarely exhibited bacterial or fungal contamination. When nystatin was only present in the initial wash, 15–25% of the explants had fungi. If nystatin were present continuously in the medium, only 4–8% of the cultures were infected. The serotonin pretreatment, in combination with moderate levels of antimicrobial agents, resulted in cultures that were visibly free of bacterial and fungal contamination. After 38 days in culture, 96% of the explants remained free of bacteria.

Once pretreated with serotonin, gill explants remained viable and free of bacteria for over a month. Neither the cells in the explant nor detached cells showed any uptake of trypan blue. The gill explant is lined with a ciliated epithelium organized into filaments. Filament organization remained unchanged (Fig. 1), and ciliary and muscular activity continued throughout the life of the explant. Both muscular and ciliary activity were altered by re-in-

troducing serotonin into the culture medium, and the effects were the same as those seen in freshly isolated gills. That is, ciliary activity increased and was more coordinated, and the gill musculature was relaxed by serotonin treatment. Such treatment dilates the openings to the water canals in the gills (Gardiner *et al.*, 1991). Thus, physiological responses to a known effector of gill activity still occur in long-term explant culture.

Media development

Various media and supplements to those media have been examined for their ability to improve short-term gill culture (Table I). Osmolarity, mussel blood, vitamin solutions, and insulin are important factors in such considerations. For maintenance of gill explants for a week or less, both diluted Ham's F-12 and AMH were acceptable. At 10 days, some explants in Ham's F-12 showed increased cellular detachment, and at two weeks, ciliary activity was reduced or non-existent. AMH medium maintained explants for over 30 days in culture, and explants exhibited coordinated ciliary activity and periodic muscular contractions. In addition, few cells detached from the explants cultured in AMH. Those explants continuously cultured with nystatin in the AMH could not be distinguished from those exposed only initially to nystatin. After 40 days in AMH culture, some explants lost their integrity; epithelial cells began to detach from explants (Fig. 1).

Metabolic viability of the cultured explants

The ability of the cultures to incorporate ^{3}H-leucine into TCA-precipitable protein was maintained at stable levels for 30 days in culture. In a representative series of *A. grandis* gill tissues cultured for 10, 24, or 31 days, incorporation of ^{3}H leucine was 5.73 ± 0.41 (n = 17) CPM (μg protein $\cdot$ h)$^{-1}$, and there was no significant difference between the three groups of gills (ANOVA, $P > 0.05$). Beyond 30 days, the viability of the gill explants declined in both *A. grandis* and *L. subrostrata,* as indicated by reduced leucine incorporation. In another study of *A. grandis* gills, leucine incorporation remained essentially constant through 44 days of culture, but declined 50% in gills cultured for 54 days.

Discussion

Explants of freshwater mussel gill can be cultured under aseptic conditions, with minimal exposure to antimicrobial agents, by treating the gill in the initial decontamination stage with serotonin in combination with antibiotic and antifungal agents. Serotonin relaxes musculature in a number of molluscan systems (Twarog, 1954; Cambridge *et al.*, 1959; Kobayashi and Hasimoto, 1982). When applied to freshwater mussel gill, exogenous serotonin relaxes the branchial musculature, dilating the water canals leading into the central water channels. This effect, combined with an increased and more synchronous ciliary beat, maximizes fluid flow through the gill. These physiological effects of serotonin on gill explants occur within seconds (Gardiner *et al.*, 1991). Incubating gills in a combination of serotonin and moderate concentrations of an antibiotic mixture for 45 min virtually eliminates all microbial and fungal contamination. This suggests that many microbes are normally sheltered within the water spaces of the gill. One of the advantages of the serotonin treatment is that aseptic cultures can be established at lower concentrations of antibiotic and antifungal agents. These cultures can be maintained in an aseptic state with low levels of antibiotics, or even none. Freedom from the confounding results of antibiotics makes gill cultures far more suitable for physiological study.

Our initial attempts to achieve aseptic cultures of freshwater mussel gills were mainly directed at elimination of the microorganisms by increasing the variety of antibiotics and elevating their concentrations. The assumption in this approach is that microorganisms are "resistant" to the antibiotics being used. This is not the case. As observed in this study, our initial failure to establish sterile cultures was due to the tissue harboring microorganisms and fungal spores in compartments that were partially isolated from the antibiotics. Perhaps a principal reason for difficulty in establishing any aseptic organ culture is the complex architecture of the organ, which provides microenvironments relatively free of antibiotics.

Elevating the concentration of antibiotics may actually be detrimental to the tissue explant. Amphotericin B proved ineffective in controlling fungal contamination, and higher concentrations were toxic to the gill explants. High concentrations of Am-B resulted in abnormally high mucous secretion and cessation of ciliary activity. Thus, while fungal contamination is an important consideration in establishing long-term explants of freshwater mussel gill, Am-B should be avoided.

We can now routinely culture viable gills for over a month. Freshwater mussel gills will survive in a variety of media for short periods. Several functions, including explant structural integrity, ciliary beat, muscular reflex activity, and a continued ability to respond to re-addition of serotonin, all indicate that the explants are functioning in organ culture. Trypan blue is excluded from the majority of cells associated with the explant whether in place or detached. However, longer-term gill culture is apparently affected by medium composition and, as yet, unknown deficiencies. The established explant cultures are useful for the study of many events associated with gill

cellular activity (*e.g.*, calcium concretion synthesis, cell membrane ion channel characteristics).

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